Ribonuclease of Bovine Milk: Purification and Properties

INTRODUCTION

The ribonuclease of bovine milk is resistant to heat at 90°C, pH 3.5, and has a pH optimum of 7.5 (2). These properties suggest a similarity to the pancreatic ribonuclease. As milk is a very rich source of ribonuclease, it seemed worthwhile to purify the enzyme and compare its properties to those of pancreatic ribonuclease.

Crystalline pancreatic ribonuclease, when chromatographed on Amberlite-IRC-50, shows two components (3). Ribonuclease A is the major component and accounts for 90% of the ribonuclease activity. Ribonuclease B is the small peak which precedes ribonuclease A and has been shown to be heterogeneous (4, 5).

Two ribonucleases have been identified in milk and have been compared with the pancreatic enzymes. This report will present evidence that the major milk ribonuclease is identical to pancreatic ribonuclease A.

MATERIALS AND METHODS

Unpasteurized skim milk was obtained from a local dairy. Pancreatic ribnuclease A and ribonuclease B were purchased from Worthington Biochemical Corporation.[2] Crystalline pancreatic ribonuclease was purchased from Sigma Chemical Company.[2]

The IRC-50 was a Rohm and Haas product—Amberlite CG-50.[2] For batchwise adsorptions it was converted to the ammonium form by the method of Morrison *et al.* (6). For column chromatography the IRC-50 was prepared as the sodium form by the procedure of Hirs *et al.* (3).

Protein Determination

Protein was estimated from absorbance at 280 mμ in a Beckman DU spectrophotometer, using 1 cm quartz cells. An absorptivity of 10 was considered to be a 1% solution.

Ribonuclease Activity

The assay method has been described by Eichel *et al.* (7). Results are expressed as milligrams of ribonuclease with crystalline pancreatic ribonuclease (Sigma Lot R718-202) used as a standard of comparison.

Column Chromatography

Columns of IRC-50 in 0.2 *M* phosphate buffer (pH 6.47) were operated according to the procedure of Hirs *et al.* (3).

Zone Electrophoresis

The electrophoretic pattern of ribonuclease was examined using cellulose acetate electrophoresis at pH 8.6 in Veronal buffer, ionic strength = 0.05 *M*. Approximately 10 μl of a 5% protein solution was placed on the cellulose acetate strip 2.5 cm from the positive pole. A potential of 200 volts was applied for 100 minutes with a current of 2 mA per strip. The method of Mhatre *et al.* (8) was followed.

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[1] Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

[2] It is not implied that the U. S. Department of Agriculture recommends products of companies mentioned to the possible exclusion of others in the same business.

AMINO ACID ANALYSIS

Samples of 4 mg in 1 ml distilled 6 *N* HCl were heated at 110°C in evacuated sealed tubes for 70 hours. Amino acid analyses were performed in an automatic amino acid analyzer by the method of Spackman, Stein, and Moore (9).

We are greatly indebted to Dr. W. G. Gordon of this laboratory for performing the amino acid analyses and for his expert interpretation of the results.

PURIFICATION PROCEDURE

STEP 1: PRECIPITATION OF CASEIN

Twenty-five gallons of milk (25°C) was adjusted to pH 4.6 by the addition of approximately 3 liters of 1.2 *N* HCl. The casein was removed by filtration through flannel bags.

STEP 2: $(NH_4)_2SO_4$ PRECIPITATION

The whey (85 liters) was adjusted to pH 6.0 by the addition of 1.6 *N* NH_4OH. Solid $(NH_4)_2SO_4$ was added until the solution became 50% saturated. The solution filtered overnight. More solid $(NH_4)_2SO_4$ was added to the filtrate until the solution reached 80% saturation. The solution was stirred for 30 minutes and filtered overnight through Whatman No. 50 filter paper.

STEP 3: ACID PRECIPITATION

The precipitate was dissolved in approximately 4½ liters of water and the pH was adjusted to 3.0 by the slow addition of 1 *M* H_2SO_4. The solution was filtered and the precipitate discarded. The filtrate was adjusted to pH 6.5 with 1 *M* NaOH.

STEP 4: IRC-50 ADSORPTION

The solution was dialyzed in the cold against distilled water for 12 hours. IRC-50 (10 gm/liter) in the ammonium form was added to the dialyzed solution. After the mixture was stirred for 2 hours, the IRC-50 was collected on a sintered glass filter and was washed with copious amounts of distilled water. The ribonuclease was eluted from the resin using 300 ml portions of 1 *M* NH_4Ac. Three elutions removed the major portion of ribonuclease.

STEP 5: ACETONE PRECIPITATION

The combined eluates were precipitated at 4°C by the addition of acetone to 58%. The cloudy suspension was centrifuged at 2000 rpm for 10 minutes in a refrigerated centrifuge. The acetone concentration of the supernatant was raised to 75%. The precipitate, collected by centrifugation, was dissolved in water and lyophilized.

STEP 6: COLUMN CHROMATOGRAPHY

The lyophilized preparation (520 mg) was dissolved in 10 ml 0.2 *M* sodium phosphate buffer, pH 6.47. The sample was chromatographed on an IRC-50 column, 4.0 × 60 cm and eluted with 0.2 *M* sodium phosphate buffer, pH 6.47. Figure 1 shows the elution of ribonuclease from a 0.9 × 30 cm column.

The contents of the tubes from each activity peak, designated ribonuclease A and ribonuclease B, were pooled. The ribonucleases were desalted by the addition of an equal volume of the resin, Amberlite MB-1. The solution was stirred for 30 minutes. The resin was removed by filtering through a sintered glass filter and was washed, using approximately 4 volumes of water. The filtrate and washings were combined and lyophilized; 148 mg of ribonuclease A and 38 mg of ribonuclease B were obtained. Table I summarizes the purification procedure of a typical experiment.

PROPERTIES

COLUMN CHROMATOGRAPHY

The separation of milk ribonuclease into ribonuclease A and ribonuclease B is shown in Fig. 1. Crystalline pancreatic ribonuclease was chromatographed under the same conditions and shows a similar elution pattern with a third, an inactive component (Fig. 2). The crystalline pancreatic ribonuclease usually shows an inactive impurity (3). Impurities in the milk preparation were adsorbed to the column and were not eluted under these conditions.

The milk ribonuclease A and ribonuclease B obtained by chromatography were compared to pancreatic preparations by electrophoresis on cellulose acetate strips. The minor milk ribonuclease, designated B, had the same specific activity (Table I) as the ribonuclease A. On cellulose acetate electrophoresis the milk ribonuclease A moves as a single component with the same mobility as pancreatic ribonuclease A. The milk

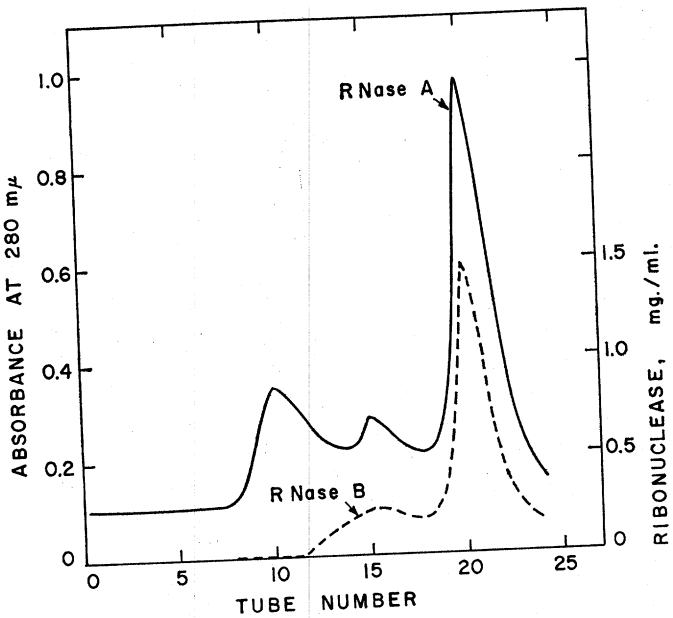

FIG. 1. Chromatography of milk ribonuclease (acetone-precipitated preparation) on IRC-50. Eight milligrams of protein was applied to a 0.9 × 30 cm column. The protein was eluted with 0.2 *M* phosphate buffer, pH 6.47. Tubes contained 1 ml. Flow rate was 1.5 ml. per hour. ——Absorbance at 280 mμ. - - - -Ribonuclease activity.

TABLE I

SUMMARY OF PURIFICATION OF RIBONUCLEASE FROM BOVINE MILK

Fraction	Volume (ml)	Total ribonuclease activity (mg)	Total protien[a] (280 mμ) (gm)	Specific activity (mg/mg)
Milk	94,600	2,000	3,300	0.000606
Whey	85,000	1,530	1,088	0.0014
Ammonium sulfate ppt. (50–80%)	6,000	822	387	0.0021
pH 3 supernatant	5,490	736	194	0.0038
Batchwise adsorption to IRC-50	620	433	1.05	0.411
Acetone ppt. (lyophilized)		378	0.521	0.726
IRC-50 chromatography				
Ribonuclease A		171	148 mg	1.16
Ribonuclease B		43.9	38.1 mg	1.15

[a] The protein for the first six analyses was based on an extinction coefficient of 10 for a 1% solution. Following chromatography an extinction coefficient of 7.1 was used (10).

ribonuclease B, on the other hand, shows two components one of which has the same mobility as ribonuclease A while the other component moves less rapidly. Electrophoresis on starch-gel gave similar results.

AMINO ACID ANALYSIS

The amino acid analysis of ribonuclease A revealed the presence of 17 amino acids which were present in the same ratio as in the pancreatic ribonuclease A (11, 12). The amino acid composition of the two enzymes appears to be identical (Table II).

IMMUNOLOGICAL STUDIES

The ribonuclease of bovine milk was studied (1) by the Ouchterlony precipitation technique with rabbit antiserum pre-

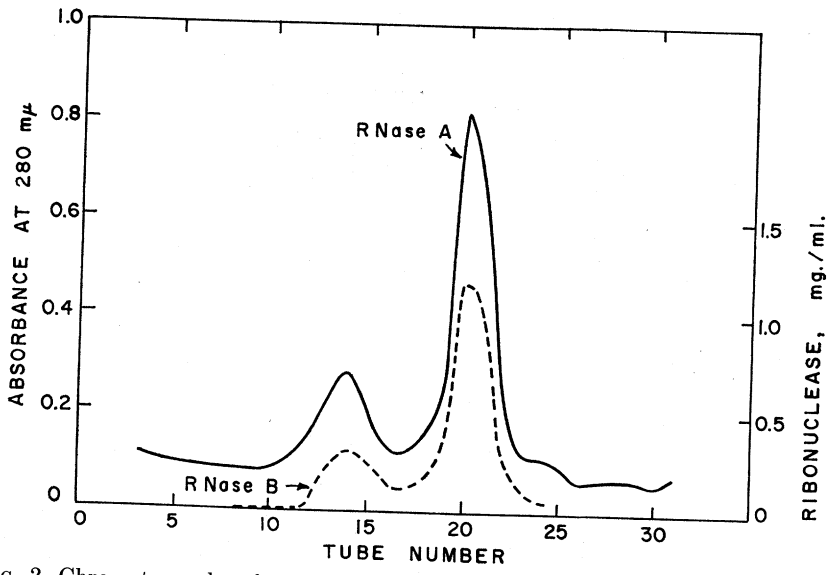

FIG. 2. Chromatography of pancreatic crystalline ribonuclease. Conditions were identical to those described in Fig. 1.

TABLE II

AMINO ACID COMPOSITION OF ACID HYDROLYZATES OF MILK RIBONUCLEASE A AND PANCREATIC RIBONUCLEASE A

The results are expressed in terms of molar ratios of the constituent amino acids, with leucine taken as 2.0 residues per molecule. The values for milk ribonuclease A are averages from two 70-hour hydrolyzates. Vaules for pancreatic ribonuclease A were obtained from Plummer and Hirs (12).

	Milk ribonuclease	Pancreatic ribonuclease A
Aspartic acid	14.2	15.0
Glutamic acid	11.2	11.9
Glycine	2.9	3.1
Alanine	11.4	12.1
Valine	8.4	9.0
Leucine	2.0	2.0
Isoleucine	2.5	2.9
Serine	12.0	10.5
Threonine	8.8	8.6
Half-cystine	6.9	6.9
Methionine	3.9	3.7
Proline	4.0	4.3
Phenylalanine	3.0	3.0
Tyrosine	5.6	5.2
Histidine	4.1	4.0
Lysine	9.7	10.2
Arginine	3.9	4.0

pared by the use of crystalline pancreatic ribonuclease. The two ribonucleases behaved identically in this test.

DISCUSSION

The results suggest that bovine milk contains only two isozymes of ribonuclease, ribonuclease A, and ribonuclease B. Pancreas zymogen granules (13), pancreatic juice (12, 13), and crystalline ribonuclease (4, 5) all show multiple components.

Plummer and Hirs (12) have recently isolated one of the minor components from pancreatic juice, which they call ribonuclease B because it chromatographs in the same position as the ribonuclease B of crystalline pancreatic ribonuclease on IRC-50. Their ribonuclease B is a glycoprotein with an amino acid composition identical to ribonuclease A. The work reported here on ribonuclease B of milk has been limited because of the small yield. However, some preliminary experiments indicate that the ribonuclease B of milk does contain carbohydrate.

The explanation for the identity of the milk and the pancreatic ribonucleases can not be decided definitely but certain possibilities appear likely. The pancreas and the

mammary gland may produce identical enzymes although this appears to be unlikely in view of genetic polymorphism due to mutations. The alternative explanation is that the pancreatic ribonuclease may appear in the milk, moving there via intestinal absorption into the blood stream followed by transmission to the milk pool in the mammary gland. Certain blood serum proteins [serum albumin and immune globulins (14–16)] do appear in the milk, and ribonuclease may follow the same pathway. Ribonuclease activity is present in blood serum (17, 18), and perhaps some or all of this may be pancreatic in origin.

If the serum ribonuclease comes from pancreatic juice then the ribonuclease must be reabsorbed through the intestinal wall into the blood stream. There is evidence that small amounts of native protein are readily absorbed from the intestinal wall (19). The low molecular weight of ribonuclease (13,600) and its resistance to proteolytic enzymes would favor this absorption.

Bovine pancreatic ribonuclease A has recently been isolated from human urine (20). Again it might be that the ribonuclease of ingested food products has been transported to the urine following absorption through the intestinal wall into the blood.

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